THE OSSIFICATION OF THE AVIAN CHONDROCRANIUM, WITH SPECIAL REFERENCE TO THAT OF THE EMU.

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(Eighteen Text-figures.)

[Read 19th August, 1942.]

I. *Introduction.*

During an investigation of "The Osteogenesis of the Base of the Saurian Cranium" recently (Kesteven, 1940*b*) it was observed that the process of ossification in the avian skull presented features of peculiar interest, not only in the region then under investigation, but in other areas as well.

It appears that there is available no detailed account of the process of ossification in any saurian skull. A careful search by myself and enquiry of my colleagues has failed to find any such. Brief descriptions of isolated stages of the normal histology of developing bone in birds, especially the chick, are to be found in the works of experimentalists studying ossification and phosphatase activity *in vitro*. These, however, do not together constitute a full account of the normal process of ossification.

The present communication is an attempt to supply the deficiency in respect of the birds.

The Emu, *Dromaeus*, has been selected for particular attention because the large size of its embryos permitted one, from the 40 mm. stage on, to dissect the skull free from all its investing tissues and so to observe the full extent of the ossified areas in each bone. This was infinitely more easy than would have been the reconstruction of similar stages of smaller embryos.

Although the description is largely based upon the Emu, the work purports to be more than just a description of the ossification of the skull in this particular bird, and unless otherwise stated, the descriptions may be accepted as applying to the birds generally. This last statement is based upon the fact that they have been found to be completely applicable, in all essential details, to any and all the other avian embryos in my collection. The essential similarity of equivalent stages observed in the numerous embryos studied, permits the assumption of equal similarity in the intervening stages, and I have not hesitated to avail myself of illustrative examples from other than emu embryos wherever it appeared desirable. Each of these is, of course, noted in the text.

The Emu has been studied in transverse and sagittal sections of the 22 mm. embryo, and transverse sections of the 37, 40, 45 and 52 mm. stages. In addition, the skulls of the 40, 45, 52, 60, 65 and 72 mm. stages have been studied in dissected specimens.*

II. The Chondrocranium. (Figs. 1, 2 and 3.)†

It was not found possible to dissect the chondrocranium of the 22 mm. stage clean of investing tissues without fracturing and tearing it, nor, on account of its size, was I confident that I could interpret correctly the van Wijhe preparation; therefore it was reconstructed from my transverse series "B".

* The lengths quoted for the embryos are in all instances the length of the head from the tip of the beak to the occiput. These lengths have been calculated from the trans-sections or measured directly from the mid-sagittal section.

A full list of the avian embryos in my collection is given in an appendix.

† The lettering on the Text-figures is as follows: *Ang.*, Angular bone; *Art. ca.*, Articular part of Meckel's cartilage; *Art. c. pal.*, Palatine branch of the cerebral artery; *Art. cer.*, Cerebral artery; *Art. mn.*, Mandibular artery; *Art. orb.*, Orbital artery; *Art. pal. l.*, Lateral palatine

As anticipated, the reconstruction revealed a close similarity to that of the 21 mm. embryo of the Ostrich, *Struthio*, as reconstructed by Brock (1937). The outlines are slightly different, particularly the sharp descent of the basicranial line behind the pituitary fossa. The outline of the mid-sagittal section of another embryo of the same age shows that this sharp descent is an exaggeration, due to distortion, either of the embryo itself or in the reconstruction, or that the angle between the basiseptal and the basicranial axes is variable at this age.*

Although the chondrocranium is so closely similar to that of the Ostrich that it does not call for detailed description, there are two features which call for special mention and discussion.

(a) The Postorbital Cartilage and the Pila Antotica.

The postorbital cartilage is, of course, simply the expanded upper portion of the pila antotica, which in its original and simplest form is a thin rod of cartilage which extends from the basal plate just in front of the otocrane to the taenia marginalis, and in using the two terms this fact should not be lost sight of. In the Emu, as in the Ostrich, the secondary attachment, which, following de Beer, Brock designates pila antotica spuria,

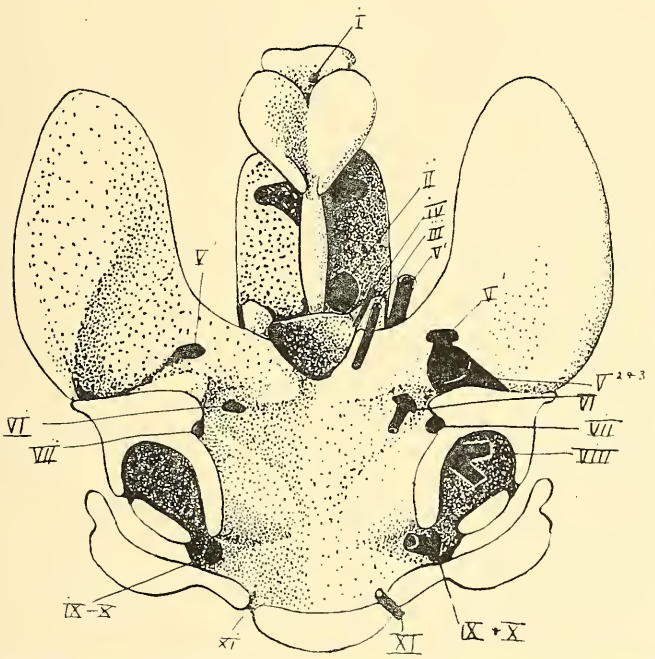

Fig. 3.—View from behind of the front part of the model of the chondrocranium of the Emu embryo (as in Figs. 1 and 2) sectioned at the line C-D.

* The reconstruction was made from outlines drawn directly onto very thick blotting-paper with the aid of a vertical projector made for me for the purpose by Esdaile and Sons of Sydney. In the outlines the nerves were included, in proximity to the cranium, and were differentially coloured. After cutting out, the outlines were cemented together with water-glass. The resulting model is very strong.

The cutting out of outlines in blotting-paper is not always a simple matter and perhaps my own method may be useful to others. I use safety-razor blades. Each blade is broken in halves and one end of the half-blade is broken to produce a fine triangular point. This is then grasped in a pair of strong artery forceps with the point projecting little more than the thickness of the blotting-paper. The forceps hold better if their grasping surfaces are clothed with a single thickness of ZO adhesive plaster. The blotting-paper to be cut is then placed upon several thicknesses of blotting-paper so that the point of the blade shall not reach the table surface if pushed deeply. When the blade point becomes blunt another little strip is broken off and a fresh point obtained. The narrow blades thus obtained turn readily in the sharpest of curves and angles, and they are, of course, very sharp and move very freely whilst cutting.

does not develop. Although the postorbital cartilage comes to lie very close to the basal plate in the situation of the spurious pila, never throughout the life of the bird is there cartilaginous continuity in that situation. From the 22 mm. stage onward two periosteal membranes alone separate the cartilages, but these membranes persist until replaced by bone between the 45 and 60 mm. stages.

Of these structures in the 21 mm. (stage 4) embryo Brock (1937, p. 232) writes: "The pila antotica spuria has grown right round the profundus nerve and is closely in contact with the pila antotica without fusing, so that the profundus has virtually a foramen separate from that of the maxillary and mandibular." Apparently, then, the conditions in the Ostrich are similar to those in the Emu; the pila antotica spuria is not completed in either bird.

Discussing the pila antotica, de Beer and Barrington (1934) write: "The next stage is represented by the fowl where the pila antotica spuria alone remains, the pila antotica disappearing, with the result that the posterior orbital cartilage appears to be connected with the basal plate by a pila which is in the wrong position for a pila antotica."

I have been unable to find the posterior orbital cartilage of the chick attached to the basal plate at any stage of development by a pila situated medial to the profundus as well as the other branches of the fifth nerve. Always the condition is as described by Gaupp (1906). I have, therefore, been led to investigate the manner of the attachment of the posterior orbital cartilage in each of the avian embryos in my collection.

De Beer and his colleagues apply the term pila antotica spuria to a secondary attachment of the first antotic process which is joined to the basal plate between the profundus and the two other branches of the fifth nerve, and they restrict the term pila antotica to the short length of cartilage by which the posterior orbital cartilage is attached to the basal plate medially to all three branches of the nerve.

The investigation of the other embryos in my collection reveals the facts that: the pila antotica spuria (1) may or may not be developed, (2) may be located behind, lateral to or in front of the pila antotica, and (3) may be greater or less than the pila antotica in the width of its attachment to the basal plate. These findings lead to the conclusion that it may be simply a secondary attachment devoid of phylogenetic significance and also that the attachment of the pila antotica, using that term to include both the postorbital cartilage and its base of attachment, is variable, and this again without phylogenetic significance.

Quoting again from de Beer and Barrington (l.c., p. 456): ". . . by following the development of this region in the duck, it is seen that all the morphological relations between the various structures are scrupulously respected, and the conditions in the fowl are nothing but the result of the development of a new structure and the reduction of an old one." It is not clear from the context, either here or in the other quoted sentence, whether the writers regarded the replacement as taking place during the life of the chick or whether they regard it as having happened in the evolution of the species. But in any case it is difficult to understand why, in view of the undeniable presence in the birds of cartilaginous structures which present all the varying relations of the alisphenoid cartilage of the mammals, it should still be necessary to regard the latter as having been developed in a totally different manner, yet de Beer (1937, p. 439) writes: "In birds the formation of the pila antotica spuria leads to the ossification of the pleurosphenoid between V_1 and V_2, but it is nevertheless a part of the true cranial wall, and has nothing to do with the alisphenoid."

With this statement the present writer most emphatically disagrees (Kesteven, 1916-1940*a*).

The varying relations of the pila antotica to the branches of the fifth nerve, which were found in the embryos examined, are schematically presented in Figure 4. It will be noted that in *Phalacrocorax* (Fig. 4D) there is a small pila antotica medial to the extensive pila antotica spuria, that in the *Iridepara* (Fig. 4G) the outer wall of the canal along which the profundus nerve runs constitutes a "spuria" slightly wider than the vera, and that in the 11·9 mm. *Podiceps* (Fig. 4F) a spurious pila has been developed in front of the true pila, whilst the latter alone is present in the 8·7 mm. embryo of this species and in the Emu (Fig. 4A).

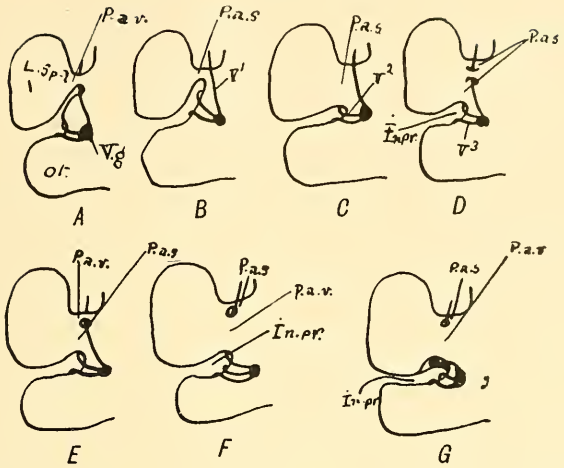

Fig. 4.—A diagrammatic presentation of the relation of the pila antotica to the branches of the fifth nerve in various avian embryos. A. Emu, all stages and *Podiceps*, 9 mm. B. Fowl and turkey, all stages, *Podargus*, 15·2 mm., *Melopsittacus*, 13th day and onwards. C. *Botaurus*, 22·2 mm. D. *Phalacrocorax*, 11·3 and 14·6 mm. E. *Apteryx*, 58·0 mm., and *Himantopus*. F. *Podiceps*, 11·9 mm. G. *Iridepara*, 4·5 and 9·7 mm. *In. pr.*, Incisura prootica; *L. sp-l.*, alisphenoidal lamina; *P.a.s.*, Pila antotica spuria; *P.a.v.*, Pila antotica vera.

[From Kesteven, 1941.]

(*b*). *The Nervus Palatinus Facialis and the Basipterygoid Process.*

Brock (l.c., p. 231) says that in the Ostrich the "palatine nerve takes its usual course ventral to the basitrabecular process". In the Emu I find that the palatine nerve leaves the ganglion immediately outside the facial canal and runs down and forward along the inner wall of the pseudotympanic cavity. It reaches the floor of that cavity immediately above the point of departure of the eustachian canal (Fig. 5, right side). Here it lies to the inner side of, and dorsal to, the internal cerebral artery just behind the point

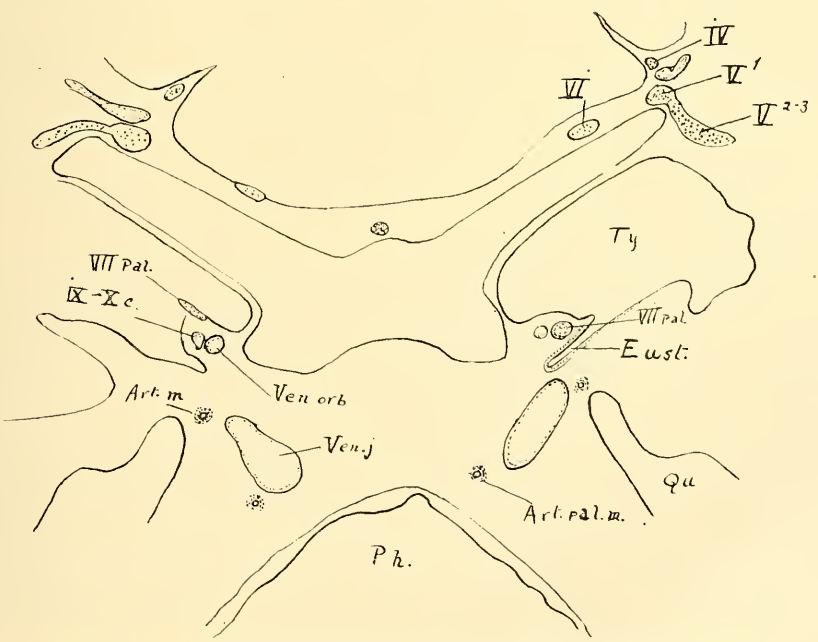

Fig. 5.—Emu, 40 mm. Transverse section behind the pituitary fossa.

where that turns dorsad and mediad to enter the lateral arterial canal. It is joined here by a nerve which was traced back to the vago-glossopharyngeal trunks (Fig. 5, left side), but which probably contains nerves from the carotid sympathetic plexus, for it carries a number of non-medullated fibres and stains more lightly than the facial nerve on that account. The fused nerves continue forward dorsally to the eustachian canal, which inclines mediad at a sharper angle. In front of the anterior wall of the cavity, which is perforated by the nerve and the accompanying palatine terminal branch of the cerebral artery, very close to the floor of the cavity, the nerve divides in two. My preparations are not stained specifically for the study of nerves, but they are sufficiently clear to permit the confident statement that there is very certainly not a separation of the original components at this division. The facial nerve comes down from above to join the other, and at the point of division it is a ventral moiety which turns ventrad and mediad to run forward below the basitrabecular process. The greater, and deeper staining, part of the nerve continues forward mediad and slightly dorsad and crosses the basipterygoid process above its inner end with the orbital vein lateral to it and the palatine artery medial to it (Fig. 6).

Briefly, it may be stated that in the Emu the palatine nerve runs dorsally to the basipterygoid process but gives off its first branch before that process is reached. It is probable that Brock mistook this first branch for the nerve itself, in the Ostrich.

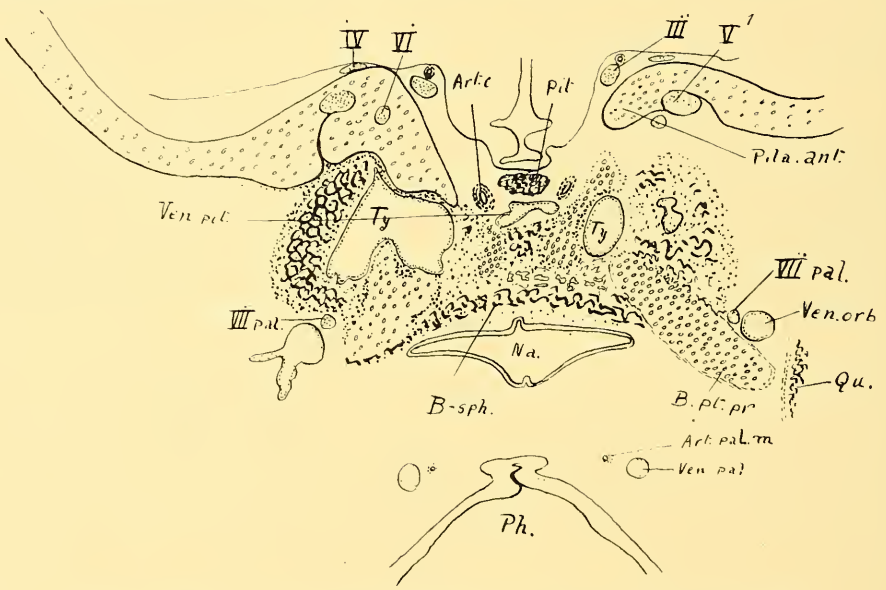

Fig. 6.—Emu, 40 mm. Transverse section in front of the pituitary fossa.

This course of the nerve seemed so aberrant that it was checked off in all the transverse series of sections and was also dissected out in a 72 mm. embryo and in a decalcified adult head, and was found to be the same in all.

Attention was then turned to the other avian embryos in my collection and I found as follows:

The only other embryos in my collection which show a basipterygoid process are the *Apteryx* and the Grebe, *Podiceps*. In the former of these the nerve branches exactly as in *Dromaeus*; in the latter the branch running below the process was not found.

In *Phalacrocorax* and in *Recurvirostra* there is a lateral projection of the postero-ventral corner of the polar cartilage which may be regarded as an abortive basipterygoid process. In both birds the nerve divides, one branch, the larger, passes dorsally accompanied by the palatine artery, the other passes ventrally.

In the other embryos it was found that the nerve always divides in the same situation and that the branch which accompanies the artery runs dorsally to the ventro-posterior corner of the polar cartilage.

It may be stated that in the birds the palatine nerve and artery pass dorsally to the basipterygoid process if that be present.

It may be stated further that in the birds the vidian canal is enclosed entirely by the basisphenoid bone, and nowhere lies in a position to be supplied with a floor by the extraperichondral bony squame which has heretofore been designated the basitemporal bone.

These two important structures present relations to the basipterygoid process very different from those they present in the lacertilians. This raises the question as to whether the avian process is in verity the same structure as that in the lizards.

At the outset of a discussion on this question it may be stated that, whilst the process in the Lacertilia is correctly designated 'basitrabecular', if equal exactitude in designation is desired in the birds the process should be termed 'basipolar'. If the polar cartilage is only a separated portion of the trabecular cartilage, this would be merely a matter of terminological exactitude and with this statement the matter might be rested. On the other hand, it is a fact that the tendency of recent writers is to regard the polar and trabecular cartilages as separate entities, and if this is correct, their processes cannot be homologous.

The polar cartilage has been observed in elasmobranchs, teleosts, birds and *Echidna*. It may, at its first appearance, be lying freely between the trabecular and parachordal cartilages, *Squalus* (van Wijhe, 1904) and *Anas* (de Beer and Barrington, 1934), or it may be attached to the parachordal, *Scyllium* (de Beer, 1931). If it be free at its first appearance it may become attached first to either the trabecula or the parachordal. In its varying relation to these two cartilages there is nothing to guide us in deciding whether it should be regarded as more nearly akin to the one or the other.

Experimental and histological evidence indicates that both the polar cartilage and the posterior end of the trabecular cartilage are of mesendodermic origin, whilst the trabecular cartilages, as far back as the oculomotor foramen, are of mesectodermic origin. Matveiev (1925) was of the opinion that the polar cartilages were of axial origin, and this belief is very strongly supported by the work of Stone (1922, 1926, 1929), Raven (1931), and Holtfreter (1933). The two former found that the trabecula behind the oculomotor foramen was not formed, like the anterior portion, from neural crest mesectoderm. The polar cartilages have not been found in the amphibian embryos they worked upon, but the portion not formed from mesectoderm is just the portion in the situation of the polar cartilage, when that is present, and it may be that this portion is in reality polar cartilage, but fuses with the hinder end of the trabecula so rapidly as to disguise its different origin.

As against this experimental evidence that the portion of the trabecula which gives rise to the basipterygoid process is of the same nature and origin as the polar cartilage, we have the opinion of Allis (1925), based upon purely theoretical reasoning, that the trabecula represents the premandibular arch and the polar cartilage the pharyngo-mandibular. This last is definitely opposed by the experimental evidence that the polar cartilages are not visceral structures. De Beer's belief (1937, p. 375) that the polar cartilages may, in some forms, represent the anterior part of the parachordals is in accord with the experimental evidence, for its origin is the same as that of the parachordal.

If, as appears probably correct, we may extend the statement of the last belief to read—the polar cartilages and the hinder end of the trabeculae are to be regarded as the anterior portion of the parachordals—then we may say of the basitrabecular process, that it is a lateral process of the anterior end of the parachordal. Such a decision calls for the abandonment of the designation basitrabecular.

However disinclined we may be to accept this recasting of our interpretation of the structures in question, in view of the facts that the basipterygoid process is developed from the polar cartilage in the birds, and the polar cartilage is certainly not part of

the trabecula, we are compelled thereto, or, in the alternative, we must regard the basipterygoid processes in the birds as not homologous with those of the reptiles.

III. Ossification.

A. General discussion.

(*a*). *Introductory.*

The description of the process of ossification which follows does not attempt a detailed account of histogenesis of the bone. I have neither the material nor the experience which would enable me to observe and/or recognize the cytological features of comparative interest and importance. On the other hand, it does attempt to present the broad outlines of the process and to describe the appearance, arrangement and fate of the different categories of elements affected by, or effecting, that process. Although the actual descriptions and drawings have been largely taken from illustrative examples provided by my collection of emu embryos the observations have been checked by the study of the other fifteen species of avian embryos in the collection. They have also been compared with the process of ossification in the embryos of ten reptilian species. In all a total of over eighty saurian embryos have been subjected to close study in the course of the work. In order to make as sure as possible that the avian conditions were being correctly interpreted, they were compared also with stages of ossification in the Urodeles, the Anura, and the caecilians, with the process of ossification in the teleosts and also with that of *Polypterus*. Finally therian conditions were studied.*

Briefly reviewing this mass of material, it may be stated that ossification of membrane bones is essentially similar throughout the series, apart from details of cellular size and structure peculiar to each particular group. On the other hand ossification of the cartilage bones presents features which are characteristic of each group.

Ossification of cartilage bones is always preceded by proliferation of the inner, osteogenetic cellular layer of the perichondrium.

In the Amphibia and the Ophidia the process is more truly endochondral than in any of the other groups, excepting the heads of long bones throughout the whole series. In these two groups ossification is ushered in by the hypertrophic degeneration of the cartilage before the proliferation of the osteogenetic layer becomes obvious, and there is little or no superficial deposition of bone before its formation in the degenerated areas of cartilage. The cartilage cells in the Amphibia are much larger than those of the Reptilia. In the reptiles (except Ophidia), in the mammals and in the Platypus ossification of the cartilage bones of the skull is invariably endoperichondral at its inception. The proliferation of the osteoblastic layer is, typically, strictly confined by the outer, membranous layer of the periosteum, and is rapidly followed by a superficial, retiform squame of bone, through the meshes of which the osteogenetic tissues invade the subjacent cartilage. The process always spreads along the surface first and only later becomes endochondral. In some reptiles this typical feature is departed from below the trabecular communi. In these forms the proliferation in this location produces a definite bulging, away from the cartilage, of the outer fibrous layer of the perichondrium and its partial or complete dissolution along a narrow strip below; simulating the condition to be described presently in the birds. In the lacertilians there is little cartilage to be ossified except in the otocrane. In the chelonians the heavier chondrocranium permits the observation of the process and it is found to differ from that of the therians in the more patchy nature of the invading process; the marked difference in the size of the cartilage cells is a striking differential feature. One also gathers the impression that, in the reptiles, calcium deposition in the cartilage does not precede that brought in by the osteoblasts, to the same extent as in the Theria. This impression is given by the lighter staining properties of the hypertrophic cartilage in the reptiles.

In the birds the ossification of the cranial cartilages is preceded by a much greater proliferation of, and activity in, the osteogenetic layer of the perichondrium than is seen in any other of the animals. In a number of situations this proliferation proceeds so far as to lead to the complete dissolution of the outer membranous layer of the peri-

* See appendix for a full list of this material.

chondrium and the invasion of the adjacent tissues by columns and clusters of osteoblasts. This leads to the formation of bone outside the limits of the perichondrium. In the following descriptions this will be designated "extraperichondral" bone or ossification.

Stump (1925), summarizing his work on the histogenesis of bone, writes: "The primitive connective tissue cells of the chondrogenetic areas in the course of growth form the chondroblasts of the cartilage, the fibroblasts of the perichondrium or the periosteum, the osteoblasts and the osteoclasts of osseus tissue, and the haemoblasts and reticular cells of marrow. They persist and extend by growth. When the cartilage model is complete they occupy a position between it and the perichondrium. It is in this region that the osteoblasts concerned with the circumferential growth of the diaphysis are evolved."

The last sentence of this quotation is a pleasing confirmation of the conclusion to which my own studies of ossification have led me. It will have been noted that Stump restricts the term perichondrium to that which I have designated—the outer layer of the perichondrium.

Whilst my own studies have led me to believe that the osteogenetic cells lie within the "perichondrium", as had been previously stated by Stump, the complete dissolution of this membrane, in numerous situations in the course of the development of the avian cranial bones, is definitely evidence that it does not always perform the only function credited to it by him, namely, that of limiting the area of ossification.

Certain fundamental facts which seem to emerge from the observations I have made may be stated as follows: Firstly, the osteogenetic cells from which "membrane" bones are developed are unquestionably recognizable as aggregations in the general mesenchyme, devoid of any limiting membrane. Clearly, neither they nor the bone they give rise to is developed from membrane. Secondly, cartilage is not "converted" into bone, it is replaced by bone. Thirdly, the perichondrium, using that term in Stump's restricted sense, takes no part in the formation of the bone, it remains unchanged throughout the process or is completely destroyed and only reformed as a periosteum after bone formation is well advanced. Clearly, the bone formed before the secondary periosteum is developed cannot have been derived from this membrane, and there is nothing in any of my preparations to suggest that the dissolution of the perichondrium was due to its conversion into osteogenetic tissue.

The terms ectochondral and endochondral should be discarded, unless in their continued use it is clearly understood that these are not different modes of ossification, but the introductory and main phases of the replacement ossification of cartilage.

It is doubtful, in view of Stump's findings, whether the terms cartilage bone and membrane bone are not misleading if they are not to be bereft of the implication that the one is derived from cartilage and the other bone from membrane.

The bald fact appears to be that bone is, in some places, developed in spatial relation to cartilage and in other places no such relation is to be found. When the spatial relation does exist, the cartilage, more or less completely, foreshadows the shape of the future bone.

Whilst this is probably the correct statement from the ontogenetic point of view, it would be foolish to deny phylogenetic significance to the spatial relation to cartilage when present.

(*b*). *General Description of the Process of Ossification in the Birds.*

1. *Cartilage Bones.*

Prior to the inception of ossification the cranial cartilages are invested by a thin, well-defined perichondrium in which the layer next to the cartilage is only just recognizably composed of cells less flattened against the cartilage than the rest. This is the osteogenetic layer of the perichondrium and the first step in the process of ossification is the proliferation of this membrane. This is initiated by the increase in thickness of the cellular layer so that it becomes recognizable at once as such (Fig. 7, 2). At this stage the fibrous layer (Fig. 7, 3) is more defined than before the inception of the changes. This phase is completed early in development almost all around the external surfaces of the cranial cartilages, and in most areas no further progress is made for some time. The next stages are observed at the primary centres of ossification. Here the

cellular layer proliferates and the osteogenetic layer becomes two and three cells deep (Fig. 7, 4). Up to this stage the cartilage appears unchanged, its peripheral cells are flattened against the perichondrium, so that in borax-carmine stained sections they simulate an inner fibrous perichondral layer (Fig. 7, 1), but in haematoxylin stained sections take the deep blue 'cartilage' colour. The cartilage cells next, within these, increase in thickness, layer by layer, till the normal outline of the cartilage cells is attained. The outer fibrous layer of the perichondrium is still intact, but has lost in staining property and in definition, both at its inner and outer surfaces. Further activity in the osteoblastic cells leads to considerable thickness in the layer and to the more or less complete dissolution of the fibrous outer membrane. In areas other than the primary centres, the initiation of further progress awaits the spread from the primary centres, when all in turn pass through the same changes.

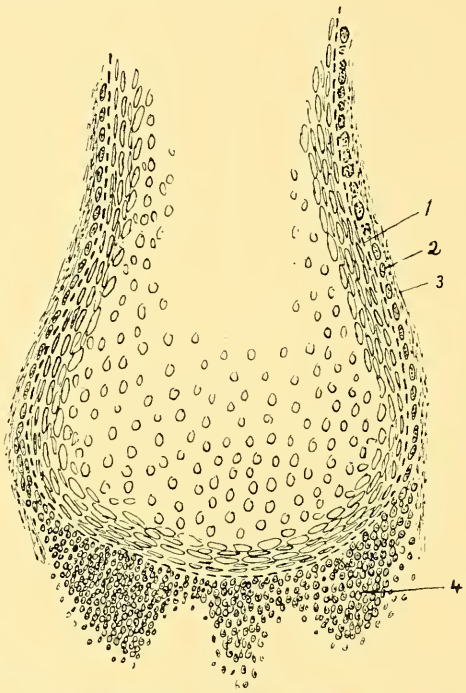

Fig. 7.—Emu, 22 mm. Transverse section through the posterior end of the intorbital septum.

The extent of the proliferation of the osteoblastic layer is very variable. In those situations where the skull is to be thickest and most extensively pneumatodic the dissolution of the fibrous outer membrane is complete, and the osteoblasts invade the surrounding tissues, so that in sections they appear in columns and clusters lying free in the connective tissue. It appears, however, that before this invasion of the surrounding tissues takes place there is a development of osteogenetic fibrils amongst the osteoblasts, because, in numerous places, a very fine, very lightly-staining fibrillar tissue is developed amongst the osteoblasts within the membranous outer layer, and before that breaks down. My want of experience and the fact that my material has been neither fixed nor stained for cytological study prevent me from stating this last as definitely as is desirable.

As development proceeds the osteoblasts are observed to arrange themselves in strands and clusters, and it is in these that the first bony spicules are deposited; the osteoblasts in the centres of the strands and clusters become imprisoned as the primary bone cells. The earliest strands to deposit bone are those in contact with the cartilage, but these are quickly followed by others further out, so that there is developed a thin layer of trabeculae of bone and/or osteoblastic strands separated and supported by a fine fibrillar tissue. At the time of the arrangement of the osteoblasts into strands and

clusters the whole osteogenetic area becomes highly vascularized by a plexus of very thin-walled vessels, these are suspended by the fibrillar tissue between the aggregations of osteoblasts.

Until the deposition of the first bony squames upon its surface there is little change in the cartilage, but just at this time its cells are found to have lost their definite boundaries and their nuclei to have become rounder and to stain more deeply. Next the cartilaginous matrix takes the stain more deeply and the nuclei lose their affinity for the stain. Deeper-staining strands of matrix soon appear and surround the cells in groups. This is the typical sequence of events, but in many places, immediately beneath the superficial squame of bone, the cartilage undergoes liquefaction without passing through these preliminary changes. The liquefaction of the cartilage commences, always, immediately next to the ectochondral bone, and from its inception one finds large polynuclear, probably syncytial, chondroclasts distributed through the liquefied area and lying against the cartilage around the area. These chondroclasts are carried out into the liquefying areas by fine osteogenetic fibrils, or travel out along those fibrils (Fig. 8).

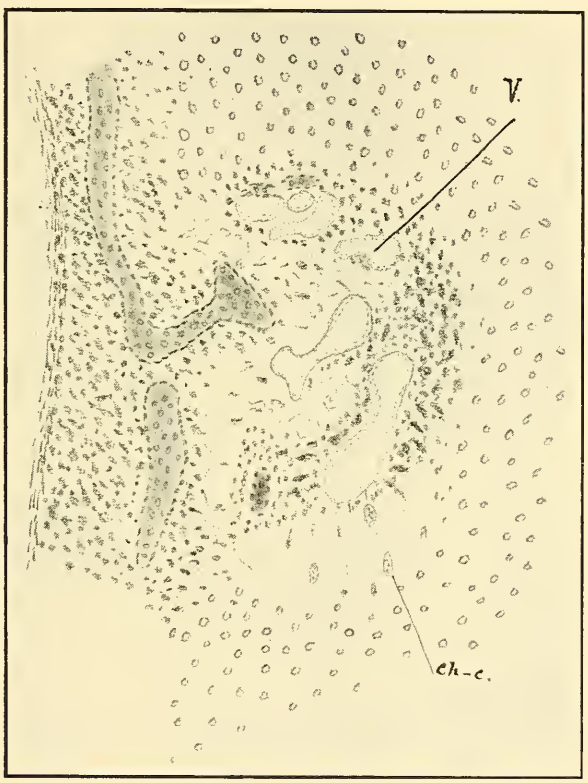

Fig. 8.—Emu, 22 mm. Section of the quadrate cartilage.

The liquefaction of the cartilage is rapidly followed by invasion of the area by osteoblasts and the vascular network from the osteogenetic area outside the investing bony squame.

This invasion of the cartilage is, of course, the inception of endochondral ossification. This proceeds on typical lines, the only feature of comparative interest of note is the fact that there is little or no columnar arrangement of the cartilage cells before the advancing osteogenetic tissue. This is probably due largely, if not entirely, to the fact that there is no growth of the cartilage in the direction of growth of the

bone, as in the ends of long bones, where this columnar arrangement of the cartilage cells is most marked. It is, however, noteworthy that even at the cartilaginous menisci in the situation of the future sutures, where cartilage growth does take place, there is little evidence of the columnar arrangement.

Where there is extension in membrane of a cartilage bone, the osteogenetic tissue for this extension is derived from the osteogenetic layer of the perichondrium along the line of outgrowth of the extension. This process differs only from the proliferation at the thickest places in the skull, in that the proliferation is much more extensive, and in that a well-marked periosteum is early developed, limiting the future bone. The actual development of the bone in these "extraperichondral" areas is similar in all respects to that already described (Figs. 11 and 12).

2. *Membrane Bones.*

The earliest sign of the membrane bones takes the form of a condensation in the mesenchyme along the tracts where these bones are to be developed. In places where membrane bones are later to make sutural contact, their early cellular stromata are, or appear to be, in complete continuity. As development proceeds the cells in these areas of condensation, or aggregation, increase in size, in number and in affinity for the basic stains. Later, but whilst the component cells of the aggregations still appear all alike, a fibrous membrane is developed around them. Almost as soon as this limiting 'periosteum' is apparent, it is found that a layer of osteoblasts, several cells deep, has become arranged against its inner surface and that numerous thin-walled capillary vessels have made their appearance in the space inside the osteoblastic layer. The central area becomes noticeably less packed with cells and very fine fibrils are present separating and suspending the capillaries and osteoblasts. Actual bone deposition is preceded by the arrangement of the osteoblasts inside the space into an open 'sponge-work' of columns and clusters. The bony trabeculae develop around the periphery and then along the columns in the central area.

3. *Comparison of the two Forms of Osteogenesis.*

Where the proliferation of the perichondrium is not so extensive as to lead to invasion of the surrounding tissue, the difference between developing membrane and cartilage bones is obvious. Where the endoperichondral proliferation leads to the dissolution of the membranous layer and the invasion of the surrounding tissues, there is a superficial resemblance between the two.

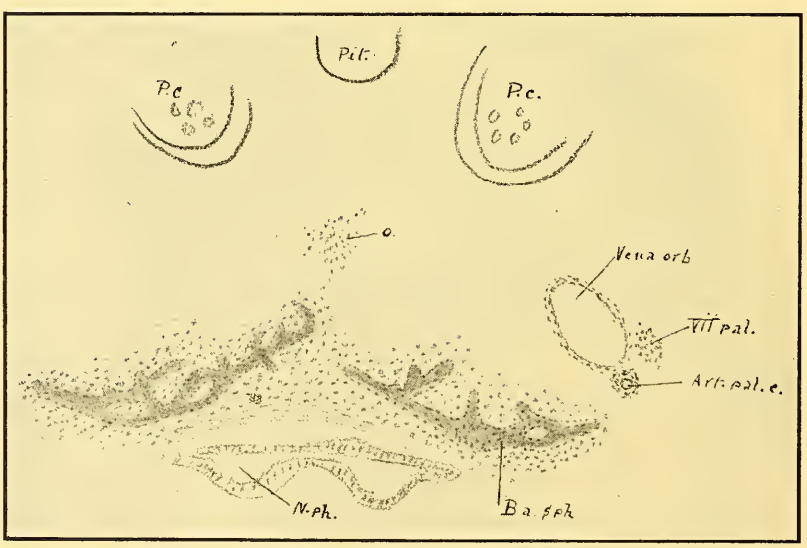

Fig. 9.—Chick, 14½ days. Transverse section through the pituitary region.

The following points of difference are therefore stressed:

The periosteum of the membrane bone is well defined, thin and stains well, and is fully developed before there is any arrangement or differentiation of the osteogenetic tissue, that of the extraperichondral bone is ill-defined, thick and lightly staining, and does not develop until after the ossification has become well advanced and actual bony trabeculae have been deposited.

Secondly, whereas there is a complete squame of bone all round the periphery of the membrane bones, in the perichondral bone the only continuous squame is that in contact with the cartilage; the free surface of these ossifying areas is found to present a multitude of trabeculae which are not gathered together to form a continuous squame, though in places their continuity does produce a small area of bone flush with the surface.

Thirdly, there is a very distinct difference in the arrangement of the osteoblasts around the free surfaces of the bones. In the membrane bone there is a dense layer several cells deep immediately inside the periosteum; in the extraperichondral bone there is not only no periosteum during the early phases of development, but also the

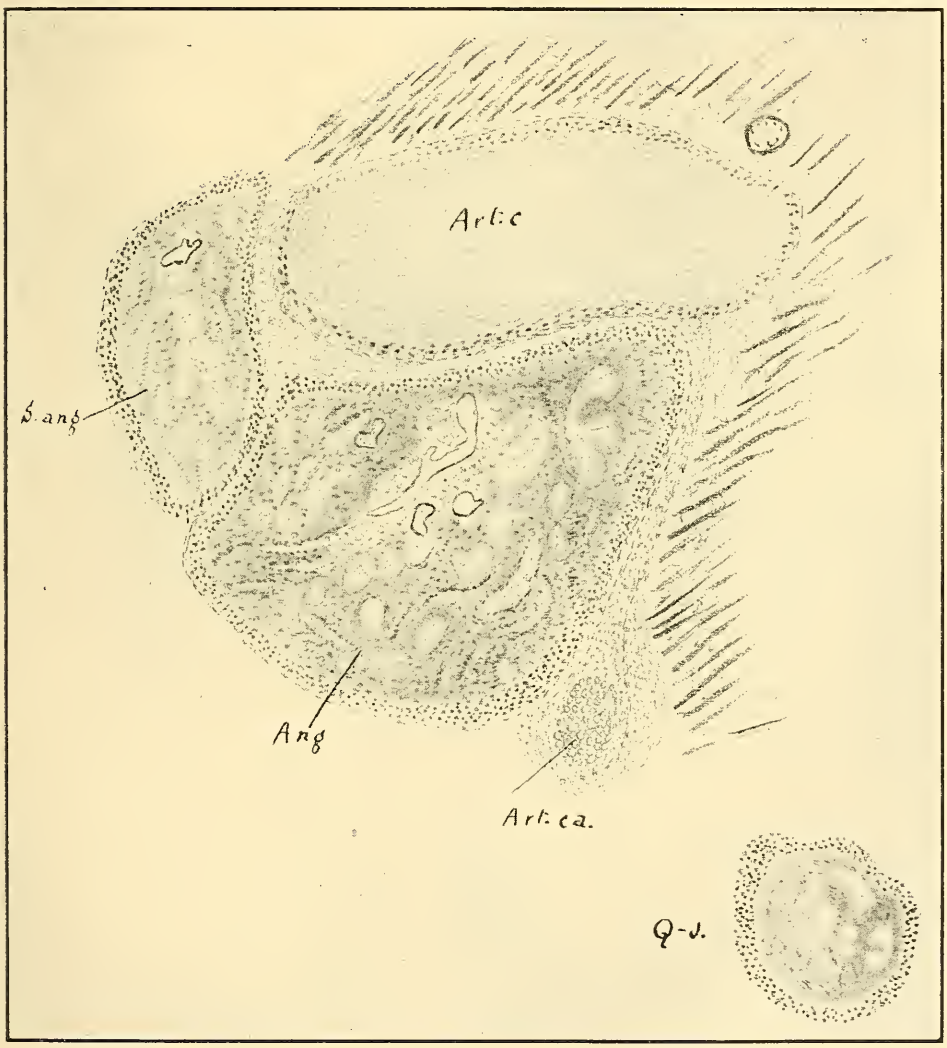

Fig. 10.—Chick, 14½ days. Transverse section through the posterior end of Meckel's cartilage.

osteoblasts at the free surface are not arranged in nearly such density, but present discontinuous columns and clusters arranged in a definite peripheral layer, but much more open in structure.

Finally, the bony squame in contact with the cartilage in the ectochondral ossification is not separated at all from the cartilage, or is separated only by a single layer of osteoblasts. In the membrane bone which lies in contact with a cartilage, as for instance the angular or surangular in contact with the articular cartilage before that ossifies, the peripheral bony squame is separated from the cartilage by two very definite membranes; its own periosteum and the perichondrium of the cartilage. These two

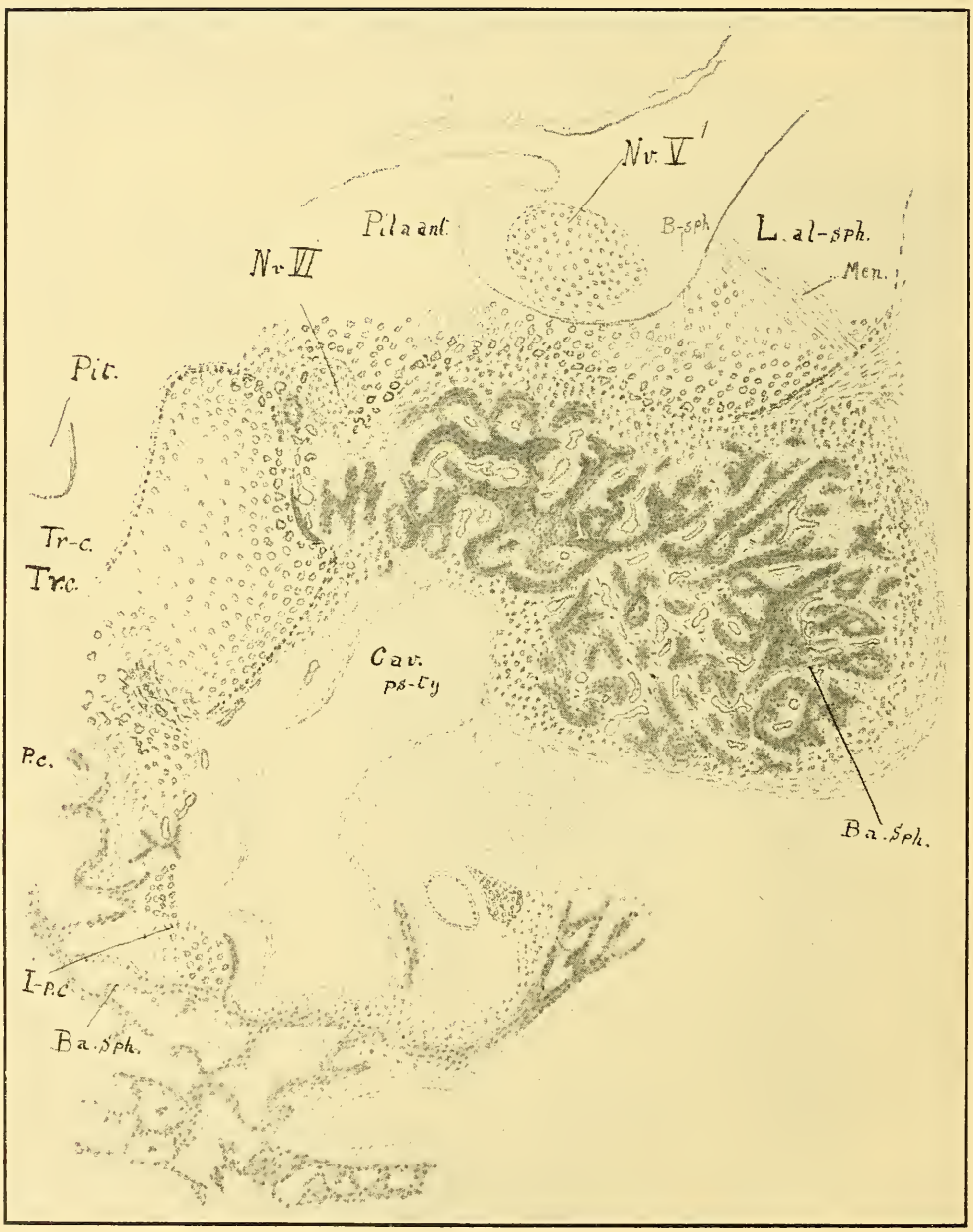

Fig. 11.—Chick, 17 days. Transverse section through the pituitary region.

membranes, or either of them, never intervene between the outgrowing perichondral bone, e.g., the ventral squame of the basisphenoid; and its cartilage.

These contrasting features are shown clearly in Figures 9 and 10. In the former the so-called basitemporal bone of the chick is depicted. The continuous squame is actually larger than is typical for such bone, but, notwithstanding this, it contrasts very strongly with the angular bone, seen in the second figure (Fig. 10) developing in close contact with the articular cartilage. Figure 11 may be compared with these. It also is taken from a chick embryo, and shows the extraperichondral bone which forms the basiphenoid bone beneath the infrapolar cartilage and that which forms the roof of the pseudo-tympanic cavity, and which later makes sutural contact with the alisphenoid bone.

(*c*). *Ossification of the Individual Bones of the Emu Skull.*

1. *The Basisphenoid.*

Ossification of this bone commences in the Emu, as in the birds generally, on the base of the skull in the neighbourhood of the pituitary fossa. Proliferation of the endoperichondrium commences just beneath the junction of the trabeculae and spreads forward beneath the intorbital septum and backwards along the ventrum of the trabeculae and polar cartilages (Fig. 12). The proliferation is extensive and leads to the complete dissolution of the membranous layer of the perichondrium and the invasion of the tissues below and between the cartilages. Very little later the same proliferation is to be seen at the dorsal edge of the trabeculae. This is a much more extensive proliferation than the other, and the osteogenetic tissue extends quite a long way out into the connective tissue. Returning to the ventral centre of ossification,

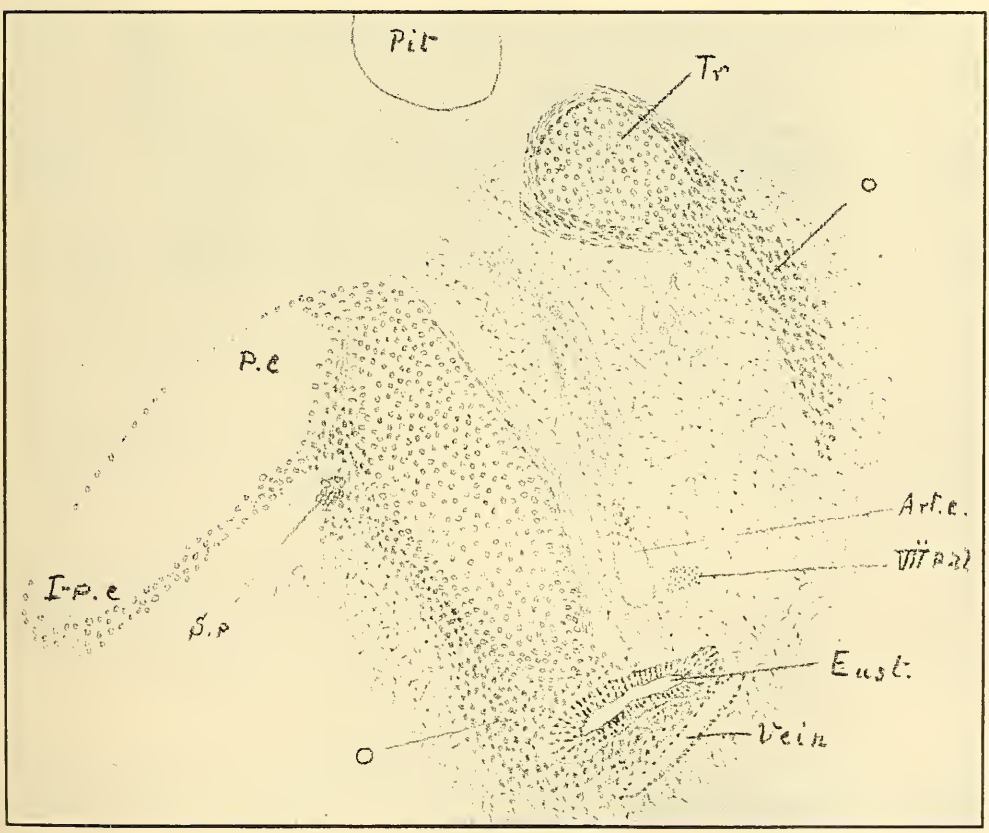

Fig. 12.—Emu, 22 mm. Section through the pituitary region.

the osteoblasts are to be seen just behind the junction of the trabeculae forming a continuous band below the floor of the pituitary fossa, lying in this situation quite freely in the connective tissue (Fig. 9).

The formation of bony trabeculae within the area of proliferation is rapid and there is developed on the base of the skull a triangular squame of bone which has its apex prolonged into a splint which runs forward under the intorbital septum, and which in its broader part behind underlies the body of the basisphenoid cartilage. Very early, however, the ossification becomes endochondral, so that the first-formed part of this ectochondral lamina is already knit to the cartilage by the invading endochondral bone a long time before the confines of the future basisphenoid bone are reached by the growing edge of the lamina. The superficial lamina grows continuously and uninterruptedly until those confines are reached and *no further*. This is important, because it is definite evidence that the lamina is in verity ectochondral bone limited by the cartilage from whose perichondrium it takes its origin, and not, as has heretofore been believed, a membrane bone. This interpretation receives further confirmation from the fact that the lamina is not confined to the ventral surface of the basisphenoid bone but is continued right around the basipterygoid process and up the sides and front of the cartilage to join the other two centres of ossification of the bone. It may be remarked that the ossification of the front of the bone is actually almost completed, in continuity with the ventral squame, whilst that lamina is little more than half its ultimate size.

To describe the progress of ossification in the other centres would be to repeat the general description of the development of the extraperichondral bone of an earlier page. Cartilage takes no place in its growth, it is developed entirely from the osteogenetic tissues proliferated from the perichondrium.

The body and the base of the bone are developed from the ventral centre. All the rest of the bone, the flanges which form the walls of the pseudotympanic cavity below the level of the squamosal contribution and in front of the otic components, is formed in membrane from these centres.

It may be remarked that the fusion of the early stromata of the basi- and presphenoid is, in my experience, the only avian example of such a fusion as between cartilage bones, but is general as between the stromata of membrane bones where these will meet in sutures in the bony skull.

In the dissected skulls of the 37 and 40 mm. embryos, it is difficult to persuade oneself that the ventral lamina is not, as has been believed, a membrane bone. It is not difficult to dissect it free from the base of the skull and it appears to have come away cleanly. Closer inspection, however, reveals the fact that there is a fracture surface between it and the endochondral bone below and around the pituitary fossa and that it has been broken away from the bony anterior wall of the front of the cartilage and the pseudotympanic cavity. In the 60 mm. skull the lamina is found to have reached all the boundaries of the basisphenoid bone, to clothe the basipterygoid process completely, to be continuous with the surface layer of the bone of the side and front walls, except where the bone makes contact with other bones, and to be firmly attached to the endochondral bone over a wide area, and no longer can it be detached without breaking it.

This bone provides an interesting example of the need to check the results of work with the scalpel and tweezers with that done with the microtome and the microscope, though, even without these, the very early and complete fusion of the lamina with the endochondral bone and the very definite limitation by the boundaries of the basisphenoid cartilage should correct the temptation to regard the bone as a parasphenoid ossification.

2. *The Basioccipital Bone.* (Figs. 13 and 14.)

This bone has but one primary centre of ossification. In the 22 mm. embryo there is a central band of proliferating perichondrium along the ventrum of the basioccipital cartilage. In the 37 mm. embryo this proliferation has increased markedly in the posterior one-third of its length. The membranous outer layer has been lost and the lower margin of the area of osteogenesis is defined by a band of osteoblasts which are

as yet devoid of any definite arrangement into columns or clusters. A small squame of bone has been deposited against the ventrum of the cartilage. Immediately above the squame there is a very small area of early disorganization of the cartilage and above this again a small area which shows increased staining of the matrix. The notochord in its canal above the latter area is degenerating (Fig. 13). In the 45 mm. embryo there

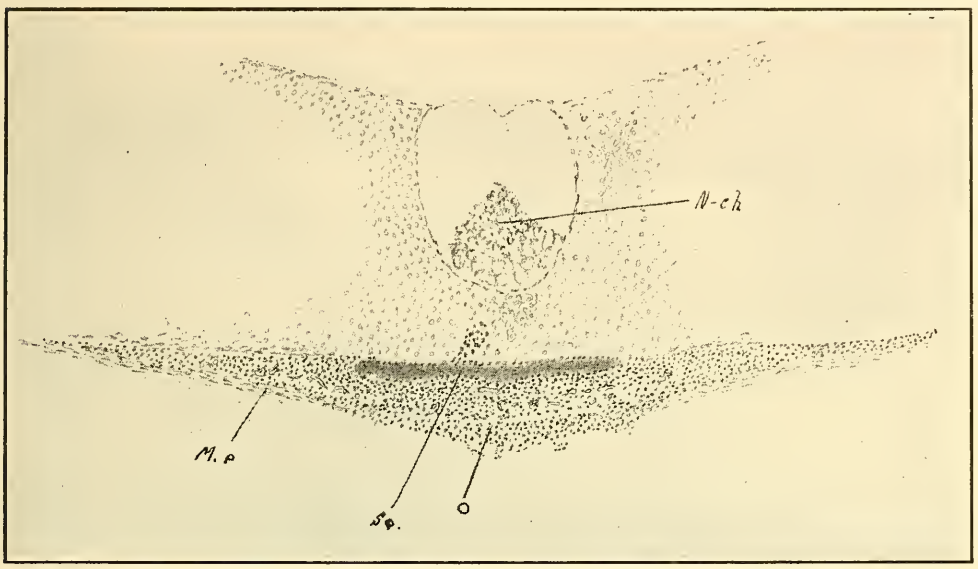

Fig. 13.—Emu, 37 mm. Transverse section through the basioccipital.

is quite extensive trabecular bone formation below the cartilage and invasion of the cartilage all round the notochordal canal and onto the dorsum of the cartilage (Fig. 14).

In the 60 mm. embryo there is an extensive area of ossification of the full depth of the bone along the centre line and extending forward for a little over half of the full length of the cartilage. In the 72 mm. embryo the bone is completed.

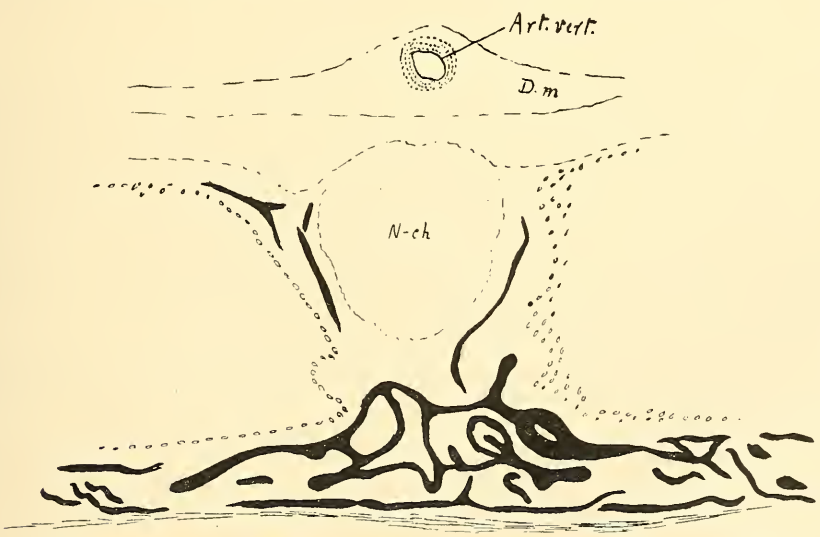

Fig. 14.—Emu, 45 mm. Transverse section through the basioccipital.

The ossification of the basioccipital presents an interesting condition intermediate between that of the basisphenoid and the exoccipital. As in the basisphenoid an extra-perichondral lamina is formed beneath the cartilage in the area invaded by the proliferation of the endoperichondrium. The lamina formed here is, however, much smaller than that of the basisphenoid. In the exoccipital bone there is no invasion of the connective tissue, and the only ectochondral lamina is that which lies in actual contact with the cartilage.

3. *The Exoccipital Bone.* (Fig. 15.)

This bone is ossified from a centre which appears close to the basioccipital at the posterior boundary of the bone. The process of ossification is essentially similar to ectochondral ossification as seen in the Theria. Proliferation of the osteogenetic layer of the perichondrium is followed almost at once by the formation of a lamina of bone against the surface of the cartilage, and this as promptly by the invasion of the cartilage by chondroclasts and osteogenetic cells. The cartilage contiguous to the centre of ossification undergoes hypertrophic degeneration, its matrix takes the basic stain and the cell nuclei lose their affinity for it. There is no disorganization of the membranous outer layer of the perichondrium.

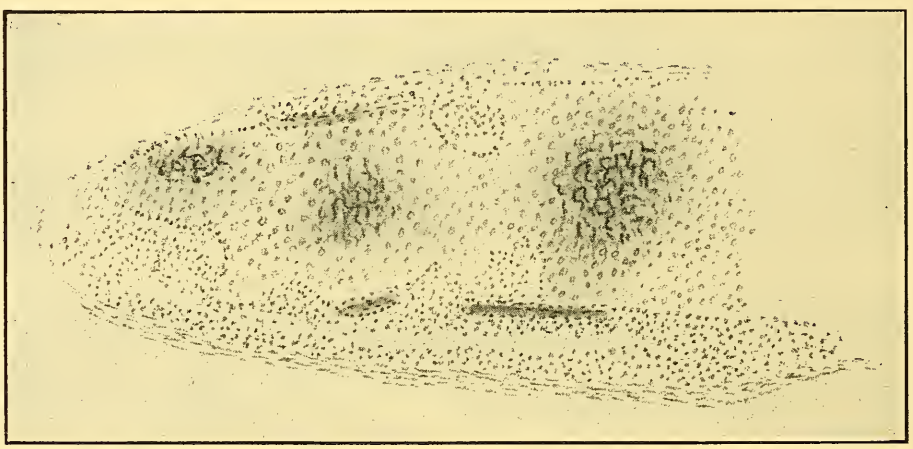

Fig. 15.—Emu, 37 mm. Transverse section through the exoccipital.

4. *The Periotic Bones.*

These bones are ossified very rapidly. In the 40 mm. embryo the only evidence of commencing ossification is the fact that the osteogenetic layer of the perichondrium is several cells deep. The membranous layer is intact. In the 52 mm. embryo three quite extensive areas of ossification are present. The smallest of these is an opisthotic centre situated laterally to the exoccipital, on the ventral surface of the otocrane, a short distance from the posterior edge. This is a perfectly typical instance of ectochondral ossification. In the 60 mm. embryo this centre has completely fused with the exoccipital ossification. This first centre of ossification in its ultimate extension surrounds most of the posterior semi-circular canal.

The second centre to appear in the otocrane is a prootic centre. It commences on the lateral surface external to the horizontal canal. This also is a typical instance of ectochondral ossification. In the 60 mm. embryo it has extended to ossify the prefacial commissure and the greater part of the vestibular portion of the capsule.

The third centre to appear is an epiotic centre, and is located on the lateral surface of the capsule externally to the upper part of the anterior canal. Like the other otocranial ossifications, this is a typical ectochondral ossification.

In the 72 mm. embryo the whole otocrane is ossified. There are cartilaginous menisci between the contacting surfaces of the prootic and the other two bones. The

epiotic, lying above the floccular fossa and surrounding only the upper portion of the anterior canal, has two of these sutures with the prootic, both at the upper margin of the fossa, one anteriorly, the other posteriorly. Menisci of cartilage separate the prootic from the posteroventral margin of the alisphenoid bone, the basisphenoid, the basi-occipital and the exoccipital bones. False sutures intervene between the epiotic and prootic and the temporal bone.

5. *The Alisphenoid Bone.**

The alisphenoid bone does not begin to ossify until rather later than the other cartilage bones. In the 52 mm. embryo the osteogenetic layer of the periosteum shows proliferative thickening in several places, particularly on the cerebral surface of the lamina alisphenoidea. In the 60 mm. embryo there are numerous small areas of invasion—liquefaction—of the cartilage, with the deposition of small trabeculae on the surface, at the edge of the areas of liquefaction and amongst the clusters of osteoblasts within the liquefied areas.

The process of ossification here differs from that of all the other cranial cartilage bones in two respects. Firstly, there are numerous small centres of ossification which are not continuous, one with the other, and the earliest of these appear on the cerebral surface of the cartilage. In all the other bones there is just one centre, or in the parasphenoid three centres, of ossification and these are on the external surface of the cartilage.

Ossification of the alisphenoid is complete in the 72 mm. embryo.

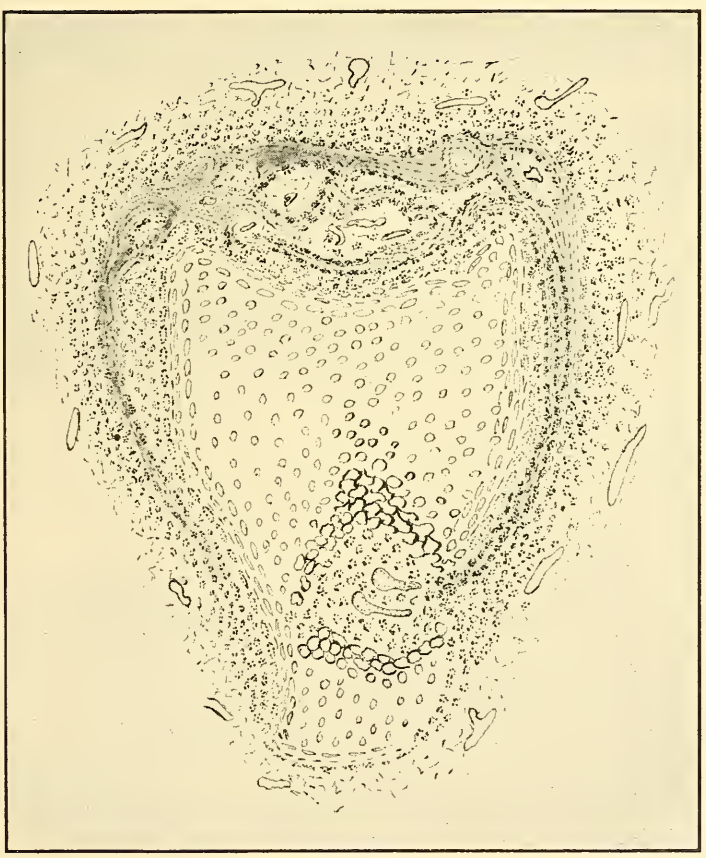

Fig. 16.—Chick, 14½ days. Transverse section through the posterior end of the intorbital septum.

* It will be observed that this designation is in conformity with the writer's belief that the bone is homologous with that of the mammals (Kesteven, 1916-1940*a*).

6. *The Presphenoid.* (Figs. 7, 16 and 17.)

The presphenoid bone is the splint which runs along under the intorbital septum and which has, in the past, been regarded as the anterior, vomerine, portion of the parasphenoid. To have found that this interpretation of the bone was an erroneous one has been one of the major surprises of this investigation. The early stages in the development of the bone have been described in the course of the general description of the ossification of cartilage bones. It is from the early stage in the development of the presphenoid bone that the illustrations of the early phases of those bones are taken.

As development proceeds the extrachondral ossification of the base of the intorbital septum increases in extent, and then the fibrous outer layer of the perichondrium at the sides of the septum appears to commence being peeled off from below upwards. This is due to proliferation of the osteogenetic layer spreading up the sides. The activity of this proliferation is much more marked posteriorly than anteriorly, both as to the height to which it reaches on the sides of the cartilage and as to the amount of bone formed beside it. There is, however, a very definite limit to the height to which it extends. Level with its highest point, all along the septum, the cartilage undergoes an interesting change. Its cells become arranged in rows with their long axes horizontal, and this layer of cartilage cells takes on the function of a growth meniscus between the presphenoid below and the septum above (Fig. 17). As development proceeds further the whole of the lower piece of the septum below this meniscus becomes ossified by invasion from the extrachondral ossification. The cartilage immediately above it

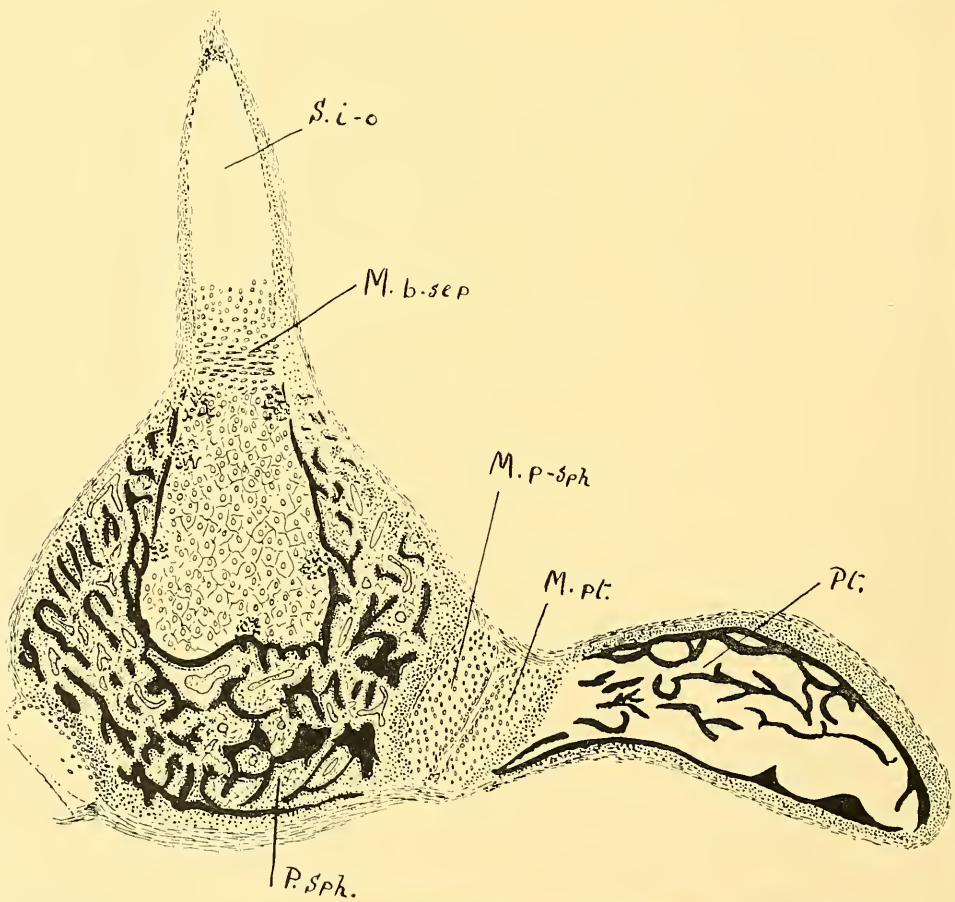

Fig. 17.—Chick, 17 days. Transverse section through the presphenoid and its articular process for the pterygoid.

remains until the intorbital septum has attained to full size, and is then ossified from the centre for the septum. Posteriorly the presphenoid bone is a relatively thick bone ossified in complete continuity with the fore end of the basisphenoid. From this point it tapers gradually to a thin splint attached to the ventral edge of the intorbital and nasal septum.

Although ultimately fused with the septum, the suture between the two remains quite well defined in young adult birds.

7. *The Intorbital and Nasal Septum.*

In the 40 mm. embryo an area of osteoblastic activity is to be found on both sides of the septum in front of and above the intorbital fenestra. In the 45 mm. embryo the full thickness of the septum has been ossified over an area which lies beneath the foramen olfactorium evehens and spreads forward to the posterior part of the nasal septum, backwards towards the intorbital vacuity and ventrally nearly half-way to the ventral edge of the septum. This ossification is brought about by typical ectochondral and replacement ossification of the cartilage. Ossification of the septum ultimately extends forward to the hinder margin of the internasal vacuity, but does not reach either the dorsal roofing bones or the ventral palatine bones in front of the posterior wall of the nasal capsule. Behind the planum antorbitale the septum is completely ossified, even the intorbital vacuity being ultimately filled in with bone.

8. *The Nasal Capsule.*

Of this only the lamina orbitonasalis, the walls of the concha, the posterior portion of the parietotectal cartilage, and just the bases of attachment of the turbinals are ossified. The manner of ossification is by typical ectochondral replacement.

9. *The Quadrate.* (Fig. 18.)

This bone begins to ossify between the 22 mm. and the 37 mm. stages. In the 37 mm. embryo the character of the ossification is clearly shown. Ossification begins in the otic process. There is a relatively marked degree of proliferation of the osteogenetic layer and the outer, membranous layer is broken through. The extraperichondral bone is not so extensive as in the central area of the basisphenoid, but is more so than in the central area of the basioccipital. The amount of this "added" bone is most marked along the medial side of the otic process and at the extremity of the quadrate between the two articular surfaces. The ectochondral ossification is soon followed by the liquefaction of the cartilage and its invasion by the osteogenetic stroma. The massive cartilage here provides remarkably fine examples of the manner of the invasion. Liquefaction is brought about by large multinucleate chondroclasts which are to be seen extruded into the liquefied area from the surface of the osteogenetic tissue. These chondroclasts are, in the majority of instances, spindle shaped, but when seen lying amongst the closely packed osteogenetic cells, they assume the most irregular shapes. Apparently the liquefied area becomes occupied by a very fine fibrillar tissue, very rapidly after the complete absorption of the cartilage. The meshes of this tissue then become filled with osteoblasts and very thin-walled capillary vessels. The arrangement of the osteoblasts in columns is followed almost at once by the appearance of fine bony trabeculae.

Amongst the osteoblasts towards the periphery of the cartilage there are numerous large multinucleate cells whose cytoplasm stains much more intensely than that of the chondroclasts and whose nuclear network is composed of thicker strands and granules of chromatin. These are believed to be osteoclasts. Their function at this stage would be, presumably, to break down the barrier to osteogenetic invasion of the cartilage, created by the deposition of the very complete squame upon its surface.

The "pterygoid" process of the quadrate ossifies very soon after the otic process. Its manner of ossification is very similar to that just described; there is, however, very little extraperichondral ossification.

The two articular regions do not ossify till very much later than the rest of the bone.

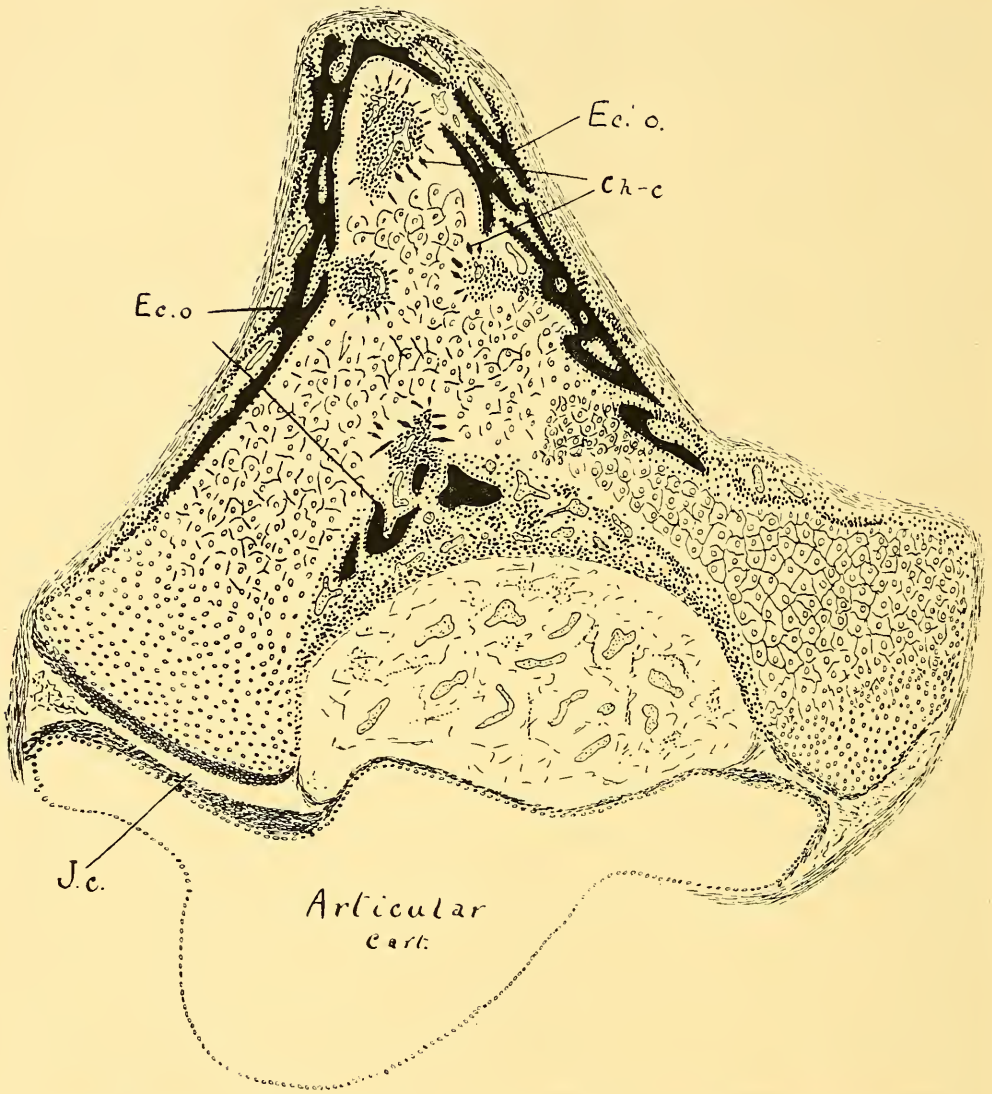

Fig. 18.—Emu, 40 mm. Transverse section through the quadrate and its articulations with the articula.

10. *The Articula.*

This cartilage does not commence to ossify until it has reached nearly adult size. I have no preparations of birds sufficiently old to enable me to describe the manner of the ossification.

11. *The Membrane Bones.*

These are all ossified earlier than the cartilage bones and in a manner so similar, one to the others, that the general description already given describes each of these, so that nothing need be added.

12. *The Articulation of the Anterior End of the Pterygoid Bone.*

The articular process for the fore end of the pterygoid bone, which is found in many birds, is developed by the extraperichondral ossification of a nodule on the side of the presphenoid bone. The outer surface of this nodule becomes clothed, late in embryonic

life, by a cartilaginous meniscus. This meniscus is probably not related genetically in any way to the cartilage of the presphenoid bone, on which the nodule is developed. This is suggested by the fact that this meniscus does not differ histologically in any way from that which is developed on the articulating surface of the pterygoid, from which it is only separated, at first by a lamina of mesenchyme and later by the joint cavity which is developed between the opposing surfaces (Fig. 18).

IV. Summary.

The ossification of the avian chondrocranium has been studied in sixteen species, and this has been compared with the ossification of the chondrocranium in a representative series of embryos of fish, amphibians, reptiles and therians.

Generalizations which emerge from the study are: (1). Ossification of cartilage bones is always a process of replacement of the cartilage by bone which is developed from osteogenetic mesenchyme, which is situated between the outer fibrous layer of the perichondrium and the cartilage, and the process of replacement ossification of cartilage always begins as an ectochondral ossification, and is completed by endochondral ossification, this last being the real replacement phase. (2). Membrane bones are not developed from membrane, but from osteogenetic cells, which appear as aggregations or condensations of cellular tissue in the general mesenchyme. (3). There is no actual ontogenetic relation between cartilage and its replacing bone, beyond the bare fact that the cartilage, at times, foreshadows the shape of the future bone. The relation between the osteogenetic tissue and the cartilage is to be regarded as a spatial one only; the bone is not developed from the cartilage.

The terms "ectochondral" and "endochondral" may be used as descriptive designations for the introductory and main phases of the complete process.

Within the birds a peculiar variety of "extraperichondral" ossification is observed in several locations around the chondrocranium. This differs from the typical ectochondral phase in that the outer, membranous layer of the perichondrium undergoes complete dissolution, and bone is developed in the adjacent mesenchyme at varying distances from the original position of the osteogenetic tissue, inside the membrane. Though a striking feature of the developing avian skull, this extraperichondral ossification is found not only amongst the Aves, but is to be observed in all vertebrates where a cartilage bone "extends in membrane".

The basitemporal bones and the parasphenoid rostrum are extraperichondral portions of the basi- and presphenoid bones respectively and not membrane bones as has heretofore been believed.

Extraperichondral ossification differs in several important and obvious features from the ossification of the membrane bones.

The basipterygoid process of the avian chondrocranium is a "basi-polar" process, but is probably homologous with that of the reptiles. The polar cartilage is certainly not a part of the trabecula. The designation basitrabecular as applied to the basipterygoid process is therefore misleading and should be discarded.

The avian alisphenoid is developed in relation to a cartilage which presents all the varying relations to the branches of the fifth nerve presented by the therian alisphenoid cartilaginous precursor; therefore both the cartilage and the bone must be regarded as homologous with the same structures in the therians.

V. *Acknowledgements.*

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I have also to acknowledge with gratitude the assistance, by way of critical discussion, the loan of the platypus embryo and references to literature, which I have received from Professors C. W. Stump and A. N. Burkitt of Sydney University.

Finally, I have to thank the Directorate of Goodyear (Australia) Ltd. for the equipment of my laboratory, and The British Australian Lead Manufacturers Pty. Ltd., of Sydney, for the specially prepared nitrocellulose, which has proven so simple to work with as an embedding mass, and which has yielded uniformly excellent sections.

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Appendix.

Materials and Method.

Besides the emu material already detailed, the following avian embryos have been studied. The lengths given are those of the head:

Acrocephalus australis Gould; 6·0 and 7·1 mm.
Apteryx; 58 mm.
Botaurus poeciloptilus Wagler; 21·2 mm.
Erythrogonys cinctus Gould; 11·0 and 12·8 mm.
Fulica atra L.; 20·1 and 22·3 mm.
Himantopus leucocephalus Gould; Ossification well advanced.
Iridepara gallinacea Temm.; 4·5 and 9·7 mm.
Melopsittacus undulatus Shaw; 13, 14, 15, 16, 17, 18 and 19 day embryos and chick.
Phalacrocorax varius Gmelin; 11·3 and 14·6 mm.
Podargus stigoides Latham; 15·2 mm.
Podiceps sp.?; 7·0, 9·0 and 11·7 mm.
Recurvirostra novaehollandiae Vieillot; 12·1 and 18·6 mm.
Thraskiornis molluca Cuvier; Ossification well advanced.
Gallus; 29, 48 and 72 hours and 9, 10, 11, 12 and 13 day embryos.
Anas; 6, 7, 8, 9, 14 and 16 day embryos.

These embryos were fixed in Bouin's solution and either stained in bulk in Grenacher's alcoholic borax carmine or on the slide in Delafield's haematoxylin. The very small embryos

were embedded in paraffin and cut at 20μ. The larger embryos were embedded in nitrocellulose and cut at 50 or 100μ with occasional sections at 25μ for histological study.

The other embryos which were studied for comparative purposes were as follows:

Fishes: *Tandanus*, *Cnidoglanis* and an unidentified Carangid. *Polypterus*.
Dipnoi: *Lepidosiren*.
Urodela: *Amphiuma*, *Amblystoma* and *Siren*.
Anura: *Hyla*, *Crinia*, *Myxophyes* and *Lymnodinastes*.
Caecilia: *Herpele*, *Dermophis*, *Geotryptes*, *Ichthyophis* and *Caecilia*.
Chelonia: *Chelodina* and *Chrysemys*.
Lacertilia: *Physignathus*, *Hinulia* (*Lygosoma*), *Tiliqua*, *Varanus* and *Diphorophus*.
Ophidia: *Python*, *Notechis*, *Pseudechis* and *Dendrophis*.
Theria: Platypus, wallaby, rat, mouse, rabbit, sheep and pig.